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Sphingolipid and Sterols of the sponge *Callyspongia spinosissima*†

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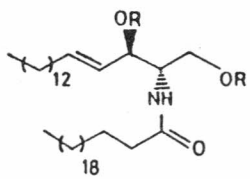
Isolation and structure elucidation of a new sphingolipid, ceramide **1** along with three known sterols cholesterol, poriferasterol and 24-methylene cholesterol as acetates from the lipid fraction of *Callyspongia spinosissima* (Dendy) are described.

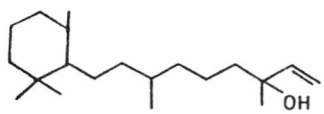
In continuation of our work on the lipoid fraction of the sponge *Callyspongia spinosissima* (Dendy) (fam. *callyspongidae*) collected from intertidal zone from the south eastern coast of India exhibiting antiviral activity[1], we report in this paper, the isolation and structure elucidation of a new ceramide **1** alongwith known sterols as their acetates. Earlier we have reported a new diterpene named callyspinol[2] **2** from the chloroform soluble fraction of the methanolic extract of the fresh sponge.

The chloroform soluble fraction of the methanolic extract after separation of the diterpene callyspinol[2] **2** was put to column chromatography and eluted with hexane-dichloromethane (4:1, v/v) when a mixture of sterols (CS-01) was obtained. Repeated column chromatography failed to yield the sterols. The EIMS of CS-01 showed cluster of molecular ion peaks at m/z 386, 398 and 412 respectively. Acetylation of CS-01 and subjecting it to preparative TLC over argentic silica plates yielded chlolesteryl acetate **3a**, porifera steryl acetate **3b** and 24-methylene cholesteryl acetate **3c** which were identified and confirmed by NMR and mass spectral studies and deacetylation to respective free sterols.

On the other hand chloroform-methanol (1:1) extract of the sponge *C. spinosissima* left after initial extraction with methanol was fractionated into acetone soluble and acetone insoluble fractions. The acetone insoluble fraction containing sphingolipids on repeated column chromatography followed by flash chromatography (vide Experimental) using chloroform—methanol (9:1) as eluent yielded a ceramide **1** as microcrystalline solid from methanol, m.p. 120°C; $[\alpha]_D^{27} - 7.0°$ [c, 0.5 $CHCl_3 - MeOH$ (1:1)], analysed for $C_{40}H_{79}NO_3$



1. R = H
1a. R = Ac



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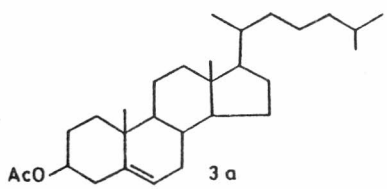
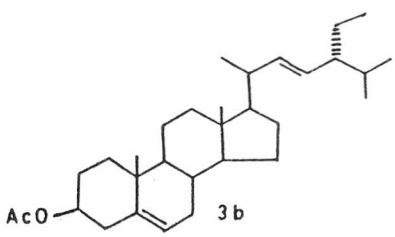
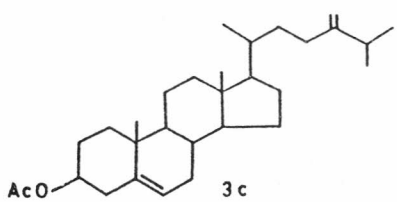


3a



3b



3c

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[^{13}C NMR and FABMS m/z 622 $(M+H)^+$]. The IR spectrum of **1** showed hydroxyl groups (3312 and 1046 cm^{-1}), an amide group (3250 and 1640 cm^{-1}), and long chain moiety (2922, 2854 and 1464 cm^{-1}) characteristic of an N-acyl sphingosine derivative. The presence of an amide group was further established from its ^{1}H NMR spectrum (400 MHz in C_5D_5N) which exhibited an −NH doublet at δ 8.4 (J=8 Hz) slowly exchangable with D_2O. This was further supported by its ^{13}C NMR spectrum showing signals at δ 175.0 and 50.0 characteristic of an amide group of ceramides. In addition to amido proton signal, the ^{1}H NMR of **1** also exhibited signals for the methyl groups of long chains as triplet at δ 0.86 (6H, J=7 Hz) and overlapping carbinol protons and methine containing amido group between δ 4.00 and 4.80 as multiplets. The presence of two olefinic proton signals was also evident at δ 5.50 and 6.68 (m). These features suggested **1** to be a ceramide nucleus. Since the ^{1}H NMR of **1** in C_5D_5N was not well resolved it was acetylated using Py/Ac_2O to yield diacetate **1a** m.p. 89°C, analysed for $C_{44}H_{83}NO_5$. The ^{1}H NMR of **1a** in $CDCl_3$ was well resolved and given in Table I. It showed the presence of two acetyl methyl signals as singlets at δ 2.0 and 2.02 thereby confirming the presence of two free hydroxyl groups. The −NH proton of the amide appeared at δ 5.68 (d, J=8 Hz) indicating secondary nature of the amide function. The two olefinic proton signals appeared at δ 5.34 (dd, J=16, 6 Hz) and 5.28 (t, J=6.8 Hz) and an amide bearing methine proton at δ 4.48 (m). The presence of 2-amido-1,3-dihydroxy-4-ene sphingosine chromophore 'A' in ceramide **1** was evident by decoupling studies carried out by irradiating proton signals at δ 4.48 which resulted in the collapse of amido proton at δ 5.68 to a singlet while two double doublets at δ 3.96 and 4.22 collapsed to doublets (J=12 Hz) and were assigned to H_{1a} and H_{1b} of sphingosine base while the triplet at δ 5.28 got changed to doublet (J=4 Hz) indicating the presence of a primary hydroxyl and a secondary hydroxyl in its neighbourhood. On the otherhand, the irradiation of proton signal at δ 5.28 resulted in the collapse of H-2 proton (multiplet) to broad double doublet while the olefinic proton at δ 5.34 collapsed to a doublet J=16 Hz, thereby, indicat-

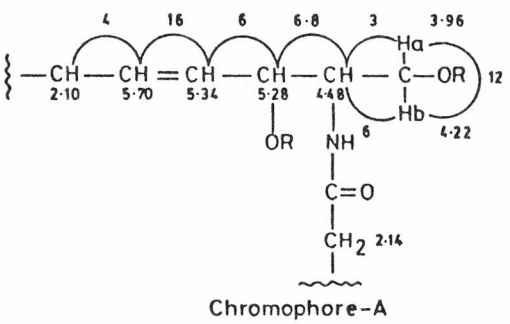

Table I—^{1}H NMR and decoupled ^{1}H NMR and ^{13}C NMR data of **1a** in $CDCl_3$ (δ, PPM, J value in Hz)

Protons	δH (J, Hz)	Decoupled signals	Carbons*	δC
Long chain base				
H-1a	3.96 (dd, 12,3)	d[a] (12)	C-1	62.60
H-1b	4.22 (dd, 12, 6)	d[a] (12)	C-2	50.40
H-2	4.48[a] (m)	—	C-3	73.70
H-3	5.28[c] (t, 6.8)	d[a] (6)	C-4	124.10
H-4	5.34[b] (dd, 16, 6)	d[c] (16)	C-5	137.30
H-5	5.70 (dt, 16, 4)	t[b] (4)	C-6	32.20
H-6	2.10 (m)	—	C-7 to C-17	29.2 to 29.6
H-18	0.86 (t, 7)	—	C-18	14.0
−NH−	5.68 (d, 8)	s[a]	$OCOCH_3\times2$	20.70
				21.00
$-OCOCH_3\times2$	2.00-2.02 ($2\times3H$)(s)		$OCOCH_3\times2$	172.2
				172.60
N-Acyl moiety			N-Acyl moiety	
H-2′	2.14 (m)		C-1′	173.10
H-3′	1.60 (m)		C-2′	36.80
H-22′	0.86 (t, 7)		C-3′	25.60
			C-4′ to C-21′	29.20 to 29.60
			C-22′	14.0

a, b and c were irradiated respectively for decoupling studies.
^{13}C assignments were based on DEPT.

ing *E* geometry of the olefinic protons leading to the presence of chromophore 'A' present in **1**.

The methanolysis of **1** with aq-methanol-H_2SO_4 yielded MFA (methylated fatty acid) which on GC-MS showed the presence of methyl docosanoate $C_{23}H_{46}O_2$ (M^+354 m/z) in 99% yields while the two other fatty acid esters methyl eicosanoate $C_{22}H_{44}O_2$ (M^+340 m/z; 0.5%) and methyl tricosanoate $C_{24}H_{48}O_2$ (M^+368 m/z; 0.48%) were minor constituents. Taking into account, the molecular weight of the ceramide m/z 621 and docosanoate as major fatty acid, the long chain base (LCB) of ceramide **1** comes out to be C-18 sphingosine base. In view of the coupling constants between H-1, H-2, H-3 in ^{1}H NMR of **1** showing 2*S*, 3*R* configuration[3], with *E* geometry for the double bond, the structure of **1** was established as (2*S*, 3*R*)-N-(docosanoate)-1,3-dihydroxy- 2-amino-octadeca-4-(*E*)-ene **1**. This was also fully in agreement with ^{13}C NMR spectrum of **1** (of Table I). This is the first example of the presence of ceramide **1** with C_{22} fatty acyl moiety and C_{18} sphingosine base in sponges in general and isolated for the first time from *Callyspoinga spinossissma* (Dendy) of Indian coasts.

Experimental Section

IR spectra in KBr were recorded on Beckmann Acculab-1-grating instrument. ^{1}H NMR and ^{13}C NMR spectra and ^{1}H NMR decoupling studies were recorded on a Bruker WM (400 MHz) instrument equipped with an Aspect 2000 computer using TMS as internal reference, mass spectra on a JOEL JMS-D-300 (70 ev) instrument and FAB MS on a JEOL 5×102 FAB instrument with JMA-DA Good Data System. Optical rotations were recorded on an Autopol III automatic polarimeter in MeOH and/or $CHCl_3$.

Animal material. The sponge *Callyspongia spinosissima* (3 Kg wet wt) was collected from intertidal zone near Rameshwaram and transported to the laboratory dipped in MeOH. A voucher specimen is preserved at the National Institute of Oceanography, Goa, India, voucher number CDR-129. The identification of the sponge was done by Dr P A Thomas, CMFRI, Trivandrum.

Extraction and isolation. The sponge material was chopped into small pieces and extracted with MeOH (3×4L) to yield methanolic crude extract A001 (29.4g). The sponge material left after extraction with methanol was extracted with $CHCl_3$-MeOH (1:1) (3×4L) to yield extract A002 (17.2g). The crude extract A001 was partitioned between $CHCl_3$ and water. $CHCl_3$ soluble fraction (F003) yielded a brown gummy mass (8.3 g) while A002 was fractionated into acetone soluble (F004) and acetone insoluble fraction (F005) which was chromatographed over silica gel column to yield fatty esters from C_6H_{12} and C_6H_{12}-CH_2Cl_2 (9:1) eluted fractions. C_6H_{12}-CH_2Cl_2 (8:2) yielded fraction 'A' and C_6H_{12}-CH_2Cl_2 (6:4) eluted fractions yielded fraction 'B' (Steroidal). Fraction 'A' on repeated chromatography using C_6H_{12}-CH_2Cl_2 gradient yielded a diterpene **2** (20 mg) purified by flash chromatography as an oil and was marked as callyspinol[2] **2** a new monocyclic diterpene. Fraction 'B' rich in sterols crystallised with methanol to yield sterol CS-01 (80 mg), m.p. 140-45°C which was a mixture of three sterols (M^+, 386, 398 and 412 m/z).

Isolation of sterols as acetates. Sterol mixture CS-01 (80 mg) was acetylated with Py/Ac_2O (1 mL Py and 1 mL Ac_2O) at r.t. leaving overnight and worked up to yield CS-01 acetate (85 mg). It was subjected to preparative TLC over silver nitrate impregnated silica gel plates run in C_6H_{12}-EtOAc (19:1) and CCl_4-CH_2Cl_2 (5:1) to yield sterol acetate-I (15.0 mg), m.p. 118-20°C, sterol acetate-II (15.0 mg), m.p. 145-47°C and sterol acetate-III, m.p. 130-32°C.

Sterol acetate-I. 3a (Cholesteryl acetate), crystalline, m.p. 118-20°; $[\alpha]_D^{27}$ −48.9° (c, 0.5 $CHCl_3$); IR (KBr): 2980, 1735, 1250 and 1030 cm^{-1}; ^{1}H NMR: δ 5.39 (brd, *J*=4.5 Hz, 6-H), 4.60 (m, 3α-H), 2.02 (s, $OCOCH_3$), 1.02 (s, 19-CH_3), 0.91 (d, *J*=7.5 Hz, 21-CH_3), 0.88 (d, *J*=7.3. 26 or 27-CH_3), 0.86 (d, *J*=6.3, 26 or 27-CH_3) and 0.68 (s, 18-CH_3); MS (EIMS): (m/z) (%) 368 (M^+-AcOH; 80), 353 (24), 260 (23), 255 (71), 247 (24), etc.

Sterol acetate-II. 3b (proifera steryl acetate), crystalline m.p. 145-47°C; $[\alpha]_D^{27}$ −58° (c, 0.5 $CHCl_3$); IR (KBr): 2980, 1730, 970 and 960 cm^{-1}; ^{1}H NMR: δ 5.38 (brd, *J*=5 Hz, 6-H), 5.14 (m, 22-H, 23-H, 24-CH_3), 4.60 (m, 3α-H), 2.02 (s, $OCOCH_3$), 1.02 (s, 19 CH_3), 0.98 (d, *J*=6 Hz, 21-CH_3), 0.82 (d, *J*=7 Hz, 27 or 26-CH_3) and 0.84 (d, *J*=6.9 Hz; 26 and 27-CH_3), 0.83 (t, *J*=7 Hz, 29-CH_3), 0.69 (s, 18-CH_3), MS (EIMS): m/z (%) 394 (M^+ − AcOH), 90, 379(9) 313(3), 310(2), 296(4), 255(8), 253(12), 228(12), 213(15), etc.

Alkaline hydrolysis (5% KOH in MeOH) of sterol acetate-II gave porifera sterol, m.p. 155-57°C; $[\alpha]_D^{27}$ +46.4; M^+ (m/z) 412 ($C_{29}H_{48}O$ requires M^+ 412 m/z).

Sterol acetate-III. 3c (24-methylene cholesteryl acetate), m.p. 130-32°C; $[\alpha]_D^{27}$ −41.8° (c, 0.55, $CHCl_3$); IR (KBr): 2980, 1748, 1255, 1040 and 870 cm^{-1}; ^{1}H NMR: δ 5.38 (b, d, *J*=Hz, 6-H), 4.60 (m, 3α-H), 4.66 (s, 28-Ha), 4.70 (s, 28-Hb), 2.00 (s, $OCOCH_3$), 1.02 (s, 19-CH_3), 0.96 (d, 9H,

J= 7.5 Hz, 21, 26 and 27-CH_3), 0.69 (s, 18-CH_3); mass (EIMS): m/z (%) 380 ($M^+ - ACOH$, 80), 368 (16), 296 (35), 281 (57%), 255 (10), 253 (33) and 213 (22).

Isolation of sphingolipid derivative: Ceramide 1. The acetone insoluble fraction of crude extract A002 on column chromatography over silica gel on elution with CH_2Cl_2 – MeOH gradient yielded ceramide **1** from CH_2Cl_2-MeOH (9:1) eluted fractions. It was further purified by flash chromatography in the same system to yield microcrystalline solid from MeOH, m.p. 120°C; $[\alpha]_D^{27} - 7.0°$ [(c, 0.5 $CHCl_3$; MeOH) (1:1)]; FAB MS (+ve mode): m/z $(M+H)^+$ 622, 608, 557, 542, 511, 490, 474, 450 etc.

Acetylation of 1 to 1a. Ceramide **1** (20 mg) was treated with pyridine (0.5 ml) and acetic anhydride (1.2 ml) and left at r.t. overnight and heated at 80°C for 2 hr. The usual work-up yielded the ceramide per acetate **1a** (20 mg), m.p. 89°C; $[\alpha]_D^{27} - 6.0$ (c, 0.4, $CHCl_3$) (^1H decoupling data given in Table I); Mass (FABMS +ve mode): m/z 706 $(M^+ + H)^+$ ($C_{44}H_{83}NO_5$ requires M^+ 705 m/z), 688, 674, 646, 643, 614 etc..

Methanolysis of 1. Isolation of methyl docosanoate and methyl eicosanote and methyl tricosanoate.

Ceramide **1** (8 mg) was refluxed with 1.2 *M* H_2SO_4 in 80% aq. MeOH for 4h. The reaction mixture was cooled and extracted with hexane. After usual processing the mixture fatty acid ester (MFA) was subjected to GC-MS analysis which showed the presence of 99% methyl docosanoate ($C_{23}H_{46}O_2$); Rt 44.00 (min); m/z 354 (M^+), 323 ($M^+ - OCH_3$), 305, 297, 283, 255 etc; 0.5% methyl eicosanoate ($C_{22}H_{44}O_2$), Rt. 41.09 (min); m/z 340 (M^+), 309 ($M^+ - OCH_3$), 283, 296, etc. and 0.48% methyl tricosanoate ($C_{24}H_{48}O_2$), Rt. 44.80 (min); m/z 368 (M^+), 337 ($M^+ - OCH_3$), 297, 283, 255 etc.

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References

1 Dhawan B N, Garg H S, Goel A K, Srimal R C, Srivastava M N & Bhakuni D S, *Indian J Exp Biol*, 31, **1993**, 505.

2 Garg H S & Agrawal Shalini, *Tetrahedron Lett*, 36, **1995**, 9035.

3 Karlsson K A, *Lipids*, 5, **1970**, 878.